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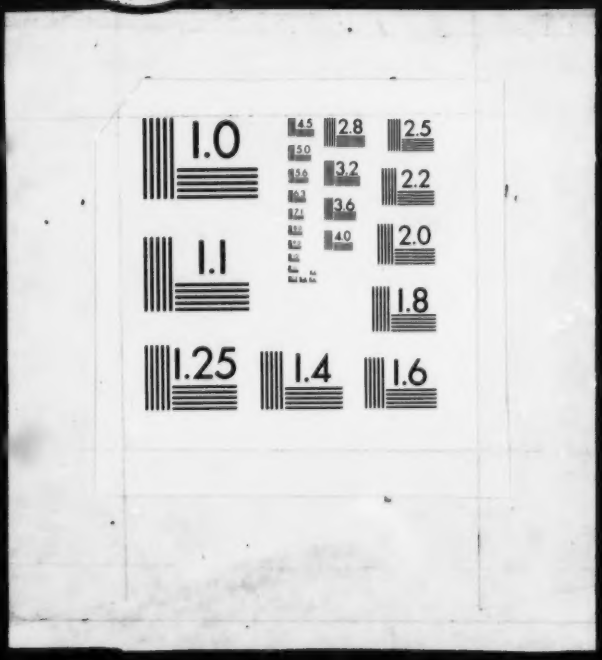

PB85-193969

# Mutagenicity of Cyclopenta-Fused Isomers of Benz(a)anthracene in Bacterial and Rodent Cells and Identification of the Major Rat Liver Microsomal Metabolites

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Nov 84

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| | |
|---|---|
| 1. REPORT NO. EPA/600/J-84/260 | 2. |
| 3. REC | PB85-193969 |
| 4. TITLE AND SUBTITLE MUTAGENICITY OF CYCLOPENTA-FUSED ISOMERS OF BENZ(a)ANTHRACENE IN BACTERIAL AND RODENT CELLS AND IDENTIFICATION OF THE MAJOR RAT LIVER MICROSOMAL METABOLITES (Journal Version) | 5. REPORT DATE 1984 |
| | 6. PERFORMING ORGANIZATION CODE |
| 7. AUTHOR(S) Nesnow, Leavitt, Easterling, Watts, Toney, Claxton, Sangaiah, Wiley, Fraher | 8. PERFORMING ORGANIZATION REPORT NO. |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS Health Effects Research Laboratory USEPA Research Triangle Park, NC 27711 | 10. PROGRAM ELEMENT NO. ACSL1A |
| | 11. CONTRACT/GRANT NO. |
| 12. SPONSORING AGENCY NAME AND ADDRESS Office of Research & Development Health Effects Research Laboratory USEPA Research Triangle Park, NC 27711 | 13. TYPE OF REPORT AND PERIOD COVERED |
| | 14. SPONSORING AGENCY CODE EPA-600/11 |

15. SUPPLEMENTARY NOTES

Published In: Cancer Research 44: 4993-5003, Nov 1984

16. ABSTRACT

The microsomal metabolites and mutagenic activity of four cyclopenta-fused benz (a)anthracenes: benz(j)aceanthrylene [B(j)A], benz(e)aceanthrylene [B(e)A], benz(l)-aceanthrylene [B(l)A] and benz(k)acephenanthrylene [B(k)A] have been studied. Aroclor-1254 induced rat liver microsomes metabolized B(j)A to: B(j)A-1,2-dihydrodiol, B(j)A-9,10-dihydrodiol, B(j)A-11,12-dihydrodiol and 10-hydroxy-B(j)A; B(e)A to: B(e)A-1,2-dihydrodiol, B(e)A-3,4-dihydrodiol, and B(e)A-5,6-dihydrodial; B(l)A to: B(l)A-1,2-dihydrodiol, B(l)A-4,5-dihydrodiol and B(l)A-7,8-dihydrodiol; and B(k)A to B(k)A-4,5-dihydrodiol and B(k)A-89-dihydrodiol. With each PAH, metabolism occurred on the cyclopentaring. All four isomers were active as gene mutagens in S. typhimurium and in Chinese hamster V79 cells. In the S. typhimurium mutation studies using Aroclor-1254 induced rat liver S9, B(j)A, B(e)A, and B(l)A required significantly less microsomal protein for maximal mutation response than B(k)A and B(a)P suggesting a one-step activation mechanism, presumably on the cyclopenta-fused ring. B(j)A B(e)A, and B(l)A were significantly more mutagenic than B(k)A and B(a)P in S. typhimurium.

17. KEY WORDS AND DOCUMENT ANALYSIS

| a. DESCRIPTORS | b. IDENTIFIERS OPEN ENDED TERMS | c. COSATI Field Group |
|---|---|---|
| | | |

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS (This Report) | 21. NO. OF PAGES 13 |
|---|---|---|
| | 20. SECURITY CLASS (This page) | 22. PRICE |

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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EPA/600/J-84/260
JOURNAL ARTICLE

[CANCER RESEARCH 44, 4993–5003, November 1984]

# Mutagenicity of Cyclopenta-fused Isomers of Benz(*a*)anthracene in Bacterial and Rodent Cells and Identification of the Major Rat Liver Microsomal Metabolites[1]

**Stephen Nesnow,[2] Sharon Leavitt, Robert Easterling, Randall Watts, Stephanie H. Toney, Larry Claxton, R. Sangaiah, Glen E. Toney, James Wiley, Phillip Fraher, and Avram Gold**

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## ABSTRACT

The microsomal metabolites and mutagenic activity of four cyclopenta-fused benz(*a*)anthracenes, benz(*j*)aceanthrylene [B(j)A], benz(*e*)aceanthrylene [B(e)A], benz(*l*)aceanthrylene [B(l)A], and benz(*k*)acephenanthrylene [B(k)A], have been studied. Aroclor 1254-induced rat liver microsomes metabolized B(j)A to B(j)A-1,2-dihydrodiol, B(j)A-9,10-dihydrodiol, B(j)A-11,12-dihydrodiol, and 10-hydroxy-B(j)A; B(e)A to B(e)A-1,2-dihydrodiol, B(e)A-3,4-dihydrodiol, and B(e)A-5,6-dihydrodiol; B(l)A to B(l)A-1,2-dihydrodiol, B(l)A-4,5-dihydrodiol, and B(l)A-7,8-dihydrodiol; and B(k)A to B(k)A-4,5-dihydrodiol and B(k)A-8,9-dihydrodiol. With each polycyclic aromatic hydrocarbon, metabolism occurred on the cyclopenta ring. All four isomers were active as gene mutagens in *Salmonella typhimurium* and in Chinese hamster V79 cells. In the *S. typhimurium* mutation studies, using Aroclor 1254-induced rat liver S9, B(j)A, B(e)A, and B(l)A required significantly less microsomal protein for maximal mutation response than B(k)A and B(a)P, suggesting a one-step activation mechanism, presumably on the cyclopenta-fused ring. B(j)A, B(e)A, and B(l)A were significantly more mutagenic than B(k)A and B(a)P in *S. typhimurium*. In the Aroclor 1254-induced rat liver S9-mediated V79 mutagenesis system, all four isomers were active, with B(l)A the most active. When Syrian hamster embryo cells were used as the metabolic activation component for V79 cells, only B(l)A produced a significant response and was equivalent in activity to B(a)P. A helical configuration for B(l)A is inferred from the identification of two *trans*-B(l)A-1,2-dihydrodiols, *syn* and *anti*, which have been synthesized, separated, and characterized. The metabolically formed dihydrodiol is *anti-trans*-B(l)A-1,2-dihydrodiol, and experimental evidence suggests that the metabolically formed B(l)A-1,2-oxide is the *anti*-isomer. Synthetic B(l)A-1,2-oxide was found to be a direct-acting mutagen in *S. typhimurium* and Chinese hamster V79 cells and is estimated to account for up to 40% of the mutagenic activity of the parent hydrocarbon. Therefore, certain cyclopenta-ring fusions on benz(*a*)anthracene appear to markedly increase its genotoxic and carcinogenic activities.

## INTRODUCTION

PAH[3] are ubiquitous environmental pollutants often formed by the incomplete combustion of fossil fuels. It is estimated that they make a large contribution to the overall carcinogenic activity of extracts of automotive emissions and other environmental contaminants to which humans are exposed; therefore, they are implicated in the etiology of human cancer (18). A unique class of PAH are the cyclopenta-fused PAH of which cyclopenta(*cd*)pyrene represents a widely studied example. Cyclopenta(*cd*)pyrene has been identified in automobile exhaust emissions, carbon black, ambient air, and cigarette smoke, is mutagenic in bacteria and mammalian cells (10, 15, 16, 35, 41, 44), induces morphological cell transformation (16), and is a mouse skin tumor initiator, cocarcinogen, and complete carcinogen (5, 6, 44). Other cyclopenta-fused PAH which have been identified in the environment, but have not yet received much attention are acenaphthylene (4, 14) and acephenanthrylene (26).

We have recently synthesized a novel subgroup of this class, the 4 isomeric cyclopenta-fused benz(*a*)anthracenes [B(j)A, B(e)A, B(k)A, and B(l)A] (36, 39). Our interest in these compounds stems from the likelihood of their occurrence in the environment [B(l)A has recently been identified as a component of wood smoke emissions],[4] and the opportunity to study the activity of the nonalternant systems generated by cyclopenta ring fusion on the benz(*a*)anthracene ring system (Chart 1). The $\Delta E_{deloc}/\beta$ values for carbocations on the cyclopenta rings of B(j)A, B(e)A, and B(l)A exceed $\Delta E_{deloc}/\beta$ of the mutagenic and carcinogenic bay region $C_{10}$ carbocation of B(a)P-7,8-dihydrodiol-9,10-epoxide (Table 1). Since carbocations of PAH with high $\Delta E_{deloc}/\beta$ values have been implicated as ultimate reactive species in mutagenesis and carcinogenesis (12, 24), it was of interest to correlate genetic activity with cyclopenta-ring metabolism in both bacteria and mammalian cells (38). Also, of interest was the extent to which the unique structure of B(l)A (Chart 1) might contribute to biological activity. This compound has a sterically crowded 4-sided peripheral indentation (gulf region) similar to the fjord region (2, 23, 30) of benzo(*c*)phenanthrene and, like benzo(*c*)phenanthrene (22), B(l)A appears to assume a helical configuration. Two sets of isomeric 1,2-oxides and *trans*-cyclopenta-ring dihydrodiols are

[1] This work was supported in part by Environmental Protection Agency Grant CR-810849 and American Cancer Society Grant BC388. Preliminary accounts of this work have appeared (33, 37).

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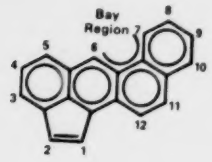
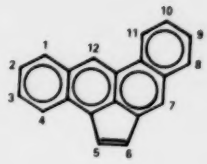
Received March 16, 1984; accepted August 13, 1984.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; B(j)A, benz(*j*)aceanthrylene; B(e)A, benz(*e*)aceanthrylene; B(k)A, benz(*k*)acephenanthrylene; B(l)A, benz(*l*)aceanthrylene; B(a)P, benzo(*a*)pyrene; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; 6TG, 6-thioguanine; SHE, Syrian hamster embryo; NMR, nuclear magnetic resonance.

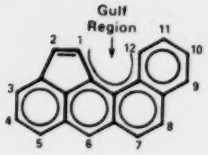
[4] R. Kamens, personal communication.



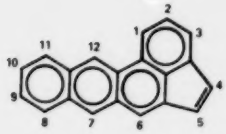

Benz(j)aceanthrylene
B(j)A



Benz(e)aceanthrylene
B(e)A




Benz(l)aceanthrylene
B(l)A



Benz(k)acephenanthrylene
B(k)A

Chart 1. Structures, nomenclature, and numbering convention of cyclopenta-fused benz(*a*)anthracenes. The bay and gulf regions are illustrated in B(j)A and B(l)A, respectively.

Table 1

*Delocalization energies ($\Delta E_{deloc}/\beta$) of the most stable benzylic carbonium ions derived from epoxides of PAH*

$\Delta E_{deloc}/\beta$ was calculated according to the method of Dewar and Dougherty (9).

| Epoxide | Carbonium ion | $\Delta E_{deloc}/\beta$ |
|---|---|---|
| B(j)A-1,2-oxide | $C_1$ | 0.879 |
| B(e)A-5,6-oxide | $C_5$ | 0.879 |
| B(l)A-1,2-oxide | $C_1$ | 0.833 |
| B(k)A-4,5-oxide | $C_5$ | 0.722 |
| B(a)P-7,8-diol-9,10-oxide | $C_{10}$ | 0.794 |

theoretically possible, and both the dihydrodiols have been identified for B(l)A.

We conclude from these studies that oxidation on the cyclopenta-ring is a primary pathway for metabolic activation of B(j)A, B(e)A, and B(l)A to forms mutagenic to *Salmonella typhimurium* and mammalian cells in culture and that these cyclopenta-ring fusions to benz(*a*)anthracene cause a dramatic increase in genetic and potentially carcinogenic activities.

## MATERIALS AND METHODS

**Synthesis of Cyclopenta-fused PAH.** Compounds were synthesized and purified by published methods (36, 39) and were shown to be pure by HPLC prior to use in metabolism experiments (38). B(l)A-1,2-oxide was synthesized and purified by published procedures describing the syntheses of related cyclopenta-fused PAH epoxides (29). A single compound was present in the reaction mixture. B(l)A-1,2-oxide was characterized by the following physicochemical data: UV (methanol) $\lambda_{max}$ ($\epsilon \times 10^{-4}$): 394 (0.140); 372 (0.168); 364 (0.200); 355 (0.233); 290 (2.37); 279 (2.29); and 269 (1.73); accurate mass of molecular ion, 268.0883; calculated for $C_{20}H_{12}O$, 268.0886 ($\Delta = -0.3$ ppm); fragmentation pattern, Table 2. $^1$H NMR (250 mHz, acetone-$d_6$) $\delta$ 5.13 and 5.85 (*d*; each 1H; J = 3 Hz; oxirane protons), $\delta$ 7.58 to 8.04 (*m*; 8H; aromatic protons); $\delta$ 8.44 (*s*; 1H; aromatic *meso*-proton $H_6$); $\delta$ 9.17 (*d*; 1H; J = 7.5 Hz; $H_{12}$). The extremely-low-field oxirane proton resonance ($\delta$ 5.85) is consistent with its location in the highly deshielded gulf region as expected for an *anti*-epoxide.[5]

*Syn-trans*B(l)A-1,2-dihydrodiol[B(l)$A_{3a}$][5] was obtained by KOH hydrolysis of B(l)A-1,2-oxide in dimethyl sulfoxide (3) and also by the sodium borohydride reduction of B(l)A-1,2-dione obtained from the selenium dioxide oxidation (20) of 1-oxo-B(l)A. A mixture of B(l)$A_{3a}$ and *anti-trans*-B(l)A-1,2-dihydrodiol[B(l)$A_{3b}$][5] was generated by sodium borohydride reduction of 1-hydroxy-2-oxo-B(l)A obtained from 2,3-dichloro-5,6-dicyanobenzoquinone oxidation (36) of a mixture of the *cis*-B(l)A-1,2-dihydrodiols (19). Dihydrodiols B(l)$A_{3a}$ and B(l)$A_{3b}$ were separated by preparative HPLC (Dupont Model 850; Dupont Instruments, Wilmington, DE) on a 9.1 mm x 25 cm DuPont Zorbax ODS reverse-phase column with a methanol-water gradient programmed as described in Chart 2*C*. The retention times were: *anti-trans*-B(l)A-1,2-dihydrodiol, 6.4 min; *syn-trans*-B(l)A-1,2-dihydrodiol, 18.9 min. The retention time of *cis*-B(l)A-1,2-diol was 17.6 min. The mass spectra of the *trans*-dihydrodiols were identical to the mass spectrum of the metabolite, *anti-trans*-B(l)A-1,2-dihydrodiol [B(l)$A_3$] (Table 2), and the $^1$H NMR traces are given in Chart 4.

*trans*-B(j)A-1,2-Dihydrodiol and *trans*-B(e)A-5,6-dihydrodiol were prepared by reduction of their respective isomeric diones. The synthesis of B(e)A-5,6-dione by the Friedel-Crafts reaction of oxalyl chloride with benz(*a*)anthracene has been reported (8). However, this report made no mention of the isolation of the isomeric B(j)A-1,2-dione whose formation could also be reasonably expected. A mixture [40% B(j)A, 60% B(e)A] of both diones in a yield of 41% by the aluminum chloride-catalyzed diacylation of benz(*a*)anthracene with oxalyl chloride in carbon disulfide was prepared. The component diones could not be separated by fractional recrystallization, HPLC, or gas chromatography, but the ratio of the components could be determined by $^1$H NMR. The dione mixture was reduced with sodium borohydride in the presence of oxygen (34) to provide a mixture of *cis*- and *trans*-B(j)A-1,2-dihydrodiol, and *cis*- and *trans*-B(e)A-5,6-dihydrodiol. This mixture was acetylated, and the *cis*-diacetate isomers were separated from the *trans*-diacetate isomers by column chromatography on deactivated alumina using hexane:methylene chloride (3:2) as the eluant. Fractional crystallization of the *trans*-diacetate mixture from hexane separated the *trans*-diacetate of B(j)A-1,2-dihydrodiol from the *trans*-diacetate of B(e)A-5,6-dihydrodiol. Each *trans*-diacetate was then hydrolyzed with dry ammonia in methanol:tetrahydrofuran (9:1), and the crude dihydrodiol recrystallized from benzene. The identity and purity of each compound was confirmed by $^1$H NMR, HPLC, UV, and mass spectroscopy. The *syn*- and *anti*-B(a)P-7,8-dihydrodiol-9,10-epoxides were obtained from the National Cancer Institute Repository.

**Generation and Collection of Metabolites.** The PAH were incubated with Aroclor 1254-induced male Charles River rat liver microsomes and a NADPH-generating system according to the previously published procedures (37, 38). A 5.0-ml incubation mixture containing 5 $\mu$mol of NADP+, 22.5 $\mu$mol of glucose 6-phosphate, 9.0 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, MO), 15 $\mu$mol of magnesium chloride, 250 $\mu$mol of potassium phosphate buffer (pH 7.5), 300 nmol of PAH, and 10.0 mg of microsomal protein was used and incubated 15 min at 37° with agitation. The incubation mixture was extracted with 15 ml of ethyl acetate:acetone (2:1). The ethyl acetate:acetone metabolite extracts were filtered (0.5-$\mu$m Teflon filter; Millipore Corp., Bedford, MA) and chromatographed by HPLC on a 6.2 mm (inside diameter) x 25 cm Zorbax-ODS column using methanol:water or methanol:water:tetrahydrofuran (3:1) gradient mixtures. Gradient programs are illustrated in Chart 2.

**Metabolite Characterization.** Separated metabolites were characterized by UV-vis spectroscopy in methanol on a Coleman 124 spectrophotometer. Electron impact mass spectra, both high resolution for elemental composition of the molecular ion and low resolution for fragmentation patterns, were obtained on a Micromass VG7070F mass spectrometer equipped with a VG 2035/B data-processing system. High-field $^1$H NMR spectra were recorded on a Bruker WM250 spectrometer. Accurate

[5] The terms *syn* and *anti* refer to the relationship between the $C_1$-$C_2$-oxirane oxygen or the $C_1$-hydroxyl group and the angular benzo-fused ring of B(l)A.

Table 2

*Mass spectral fragmentation patterns of cyclopenta-fused benz(a)anthracene derivatives*

Mass spectra were obtained by electron impact at 70 eV. The values presented represent the relative intensities.

| | *m/e* | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Metabolite | 288 | 287 | 286 | 285 | 284 | 270 | 269 | 268 | 267 | 256 | 255 | 254 | 253 | 252 | 242 | 241 | 240 | 239 | 238 | 237 | 236 |
| B(j)$A_1$ | | | 40 | | 25 | | 30 | 100 | 1 | | 20 | | | | | | 30 | 65 | | 20 | 20 |
| B(j)$A_2$ | | 26 | 100 | 5 | | | 17 | 48 | | | 24 | | | | | 16 | 24 | 34 | | | |
| B(j)$A_4$ | | 5 | 26 | | | 11 | 31 | 100 | | | 14 | | | 12 | | 9 | 40 | 68 | | 16 | 17 |
| B(j)$A_6$ | | | | | | 4 | 27 | 100 | 3 | | | | | | | 2 | 17 | 67 | 7 | 18 | 3 |
| B(e)$A_2$ | | 15 | 61 | | 5 | | 23 | 100 | 6 | 7 | 22 | 9 | | 9 | | 19 | 41 | 81 | 9 | 17 | |
| B(e)$A_3$ | | 3 | 11 | | 1 | 4 | 26 | 100 | 3 | 3 | 11 | 5 | 1 | 4 | 7 | 11 | 45 | 66 | 9 | 17 | 3 |
| B(e)$A_4$ | | 4 | 13 | | | 4 | 26 | 100 | 3 | 2 | 7 | | 1 | 3 | 4 | 9 | 33 | 52 | 7 | 14 | 2 |
| B(l)$A_3$ | 4 | 22 | 71 | 3 | 3 | 5 | 25 | 100 | 10 | 9 | 33 | 3 | 3 | 9 | 5 | 17 | 38 | 95 | 13 | 22 | 4 |
| B(l)$A_5$ | 4 | 12 | | | 7.5 | 15 | 48 | 100 | | | 15 | 12 | 15 | 23 | | 12 | 30 | 52 | 8 | 17 | |
| B(l)$A_6$ | | 23 | 60 | | 7 | 9 | 21 | 100 | 45 | 24 | 61 | | | | 9 | 49 | 73 | 100 | 12 | 24 | |
| B(k)$A_2$ | 3 | 24 | 93 | 4 | 10 | 5 | 28 | 100 | 4 | 7 | 18 | 6 | 2 | 7 | 3 | 11 | 38 | 82 | | 16 | 2 |
| B(k)$A_3$ | 2 | 11 | 42 | 3 | 5 | 5 | 30 | 100 | 4 | 6 | 17 | 3 | 2 | 9 | 13 | 17 | 62 | 70 | 8 | 15 | |
| B(l)$A_{3a,b}$ [a] | | | | | | 3 | 22 | 100 | 4 | | | | 5 | 25 | 3 | | 20 | 50 | 5 | 10 | |

[a] The fragmentation patterns and relative intensities of B(l)$A_{3a}$ and B(l)$A_{3b}$ were identical.

mass determinations and fragmentation patterns were consistent with all dihydrodiol structural assignments and with the single phenol metabolite identified (Table 2). Metabolite yields were approximately ordered by comparison of the signal:noise ratios of the $^1$H NMR spectra of samples accumulated from an equal number of semipreparative HPLC runs, made up to equal volumes in acetone-$d_6$ and generated from the same number of transients ($4 \times 10^4$).

**Bacterial Mutagenesis Assays.** The standard plate incorporation assay of Ames *et al.* (1) was used with minor modifications (7) using *S. typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100. The B(l)A-1,2-oxide and diol-epoxides of B(a)P were evaluated using the procedure of Wood *et al.* (46). The epoxide was added in 12.5 $\mu$l of anhydrous dimethyl sulfoxide to 0.5 ml of PBS, pH 7.0, which contained $2.0 \times 10^8$ bacteria. After 5.0 min at 37°, 2.0 ml of molten-top agar were added to the incubation mixture, and the solution was poured onto Petri dishes as described above.

**Cell Culture Conditions.** V79 cells were provided by Dr. E. Huberman, Argonne National Laboratory, Chicago, IL. The cells were maintained in William's Medium E supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Grand Island Biological Co., Grand Island, NY). Cultures were incubated in humidified incubators at 37° in 5% $CO_2$ in air. Cell cultures were routinely selected with hypoxanthine, aminopterin, thymine, and glycine before each experiment.

**V79 Cell Mutagenesis.** The V79 cells were plated at a density of $1 \times 10^6$ cells/25-sq cm T-flask (2 flasks/treatment group) and incubated overnight. The media were removed, and the cells were incubated in 2 ml of a reaction mixture, containing an Aroclor 1254-induced rat liver S9 fraction at the appropriate protein concentration, 0.6 ml Dulbecco's PBS (KCl, 0.2 mg/ml; NaCl, 0.2 mg/ml; NaCl, 8 mg/ml; $Na_2HPO_47H_2O$, 2.16 mg/ml) with $MgCl_26H_2O$ (0.49 mg/ml), glucose 6-phosphate (13 mg/ml), NADPH (6.3 mg/ml), garamycin (5 $\mu$g/ml; Schering Corp., Kenilworth, NJ), and PAH. The glucose 6-phosphate and NADPH were sterilized by filtration prior to use. The flasks were incubated for 4 hr at 37° in 5% $CO_2$ in air.The cells were then washed twice with PBS and refed with 5 ml of fresh media. The control cells were treated in the same manner, except culture media was used instead of reaction mixture. The cells were dispersed using 0.25% trypsin and 0.5 mM EDTA, centrifuged, and resuspended in a small volume of media. The V79 cells were counted using a hemocytometer. For determination of initial cloning efficiency, 100 V79 cells were seeded in 5 ml of complete media into 60-mm Petri dishes (5 dishes/point) and allowed to grow 6 to 7 days at which time they were fixed and stained, and colonies enumerated. For determination of 6TG-resistance, two 75-sq cm T-flasks/group were seeded at 200,000 cells/flask and allowed to grow 4 days. At this time, the cultures were reseeded again ($5 \times 10^5$ cells/75-sq cm T-flasks), and on the seventh day following treatment, the cultures were examined for cloning efficiency as described above and replated into 6TG (final concentration, 5 $\mu$g/ml) containing media ($2 \times 10^4$ cells/60-mm dish). Dishes were fixed and stained 12 to 14 days later without a media change and colonies enumerated.

Irradiated SHE cells (5000 rad) were seeded ($2 \times 10^6$ cells/75 sq cm T-flask) and incubated overnight at 37° in 5% $CO_2$ in air. The next morning, V79 cells ($3 \times 10^5$ V79 cells/flask) were seeded on top of the SHE cells. The V79 cells were allowed to attach to the SHE cells then treated with the PAH. The treatment period was 48 hr. At this time, the V79 cells were dispersed using 0.25% trypsin and EDTA, centrifuged, and resuspended in a small volume of medium. Determination of cytotoxicity and 6TG-resistance was performed as described for the S9-mediated mutation assays.

## RESULTS

**Metabolite Generation, Collection, and Identification.** To obtain metabolite mixtures for characterization, multiple 5-ml incubations were performed under conditions similar to those used in the *Salmonella* and V79 mutation studies and the extracts combined for HPLC analysis and collection. Higher product yields were obtained by this method than by large-scale incubations.

Features common to the UV and $^1$H NMR spectra of certain metabolites were useful in assigning structures. Saturation of the cyclopenta-double bond leaves the benz(*a*)anthracene chromophore. Partial saturation of the terminal benzo ring by dihydrodiol formation generates a chromophore similar to acephenanthrylene or phenanthrene, while partial saturation of the angular benzo ring produces an anthracene-like chromophore, aiding in identification of terminal and angular-ring dihydrodiols.

The protons of the highly olefinic cyclopenta-bond have a characteristic AX quartet coupling pattern (17, 36, 37, 39) in the $^1$H NMR, with $J_{AX} \approx 5$ Hz. The presence of this AX quartet in the $^1$H NMR of a metabolite is conclusive evidence that the cyclopenta-ring has not been altered. Partial saturation of a terminal ring leaves a styrene-like double bond with proton resonances at shifts characteristic of vinyl systems. Appearance of resonances in this region are helpful in confirming angular or terminal-ring dihydrodiols. Protons adjacent to the 3-sided aromatic bays of B(j)A, B(e)A, B(k)A, or the 4-sided gulf region (Chart 1) of B(l)A are highly deshielded by ring current (31) and appear at low field, well separated from other aromatic signals. The identification of such highly deshielded protons in the NMR spectra of metabolites indicates that the rings forming the bay or gulf retain aromaticity and rules out metabolite structures in which the aromaticity of bay or gulf peripheries is interrupted.

**B(j)A.** Metabolites B(j)$A_1$, B(j)$A_2$, B(j)$A_4$, B(j)$A_6$ (Chart 2) have

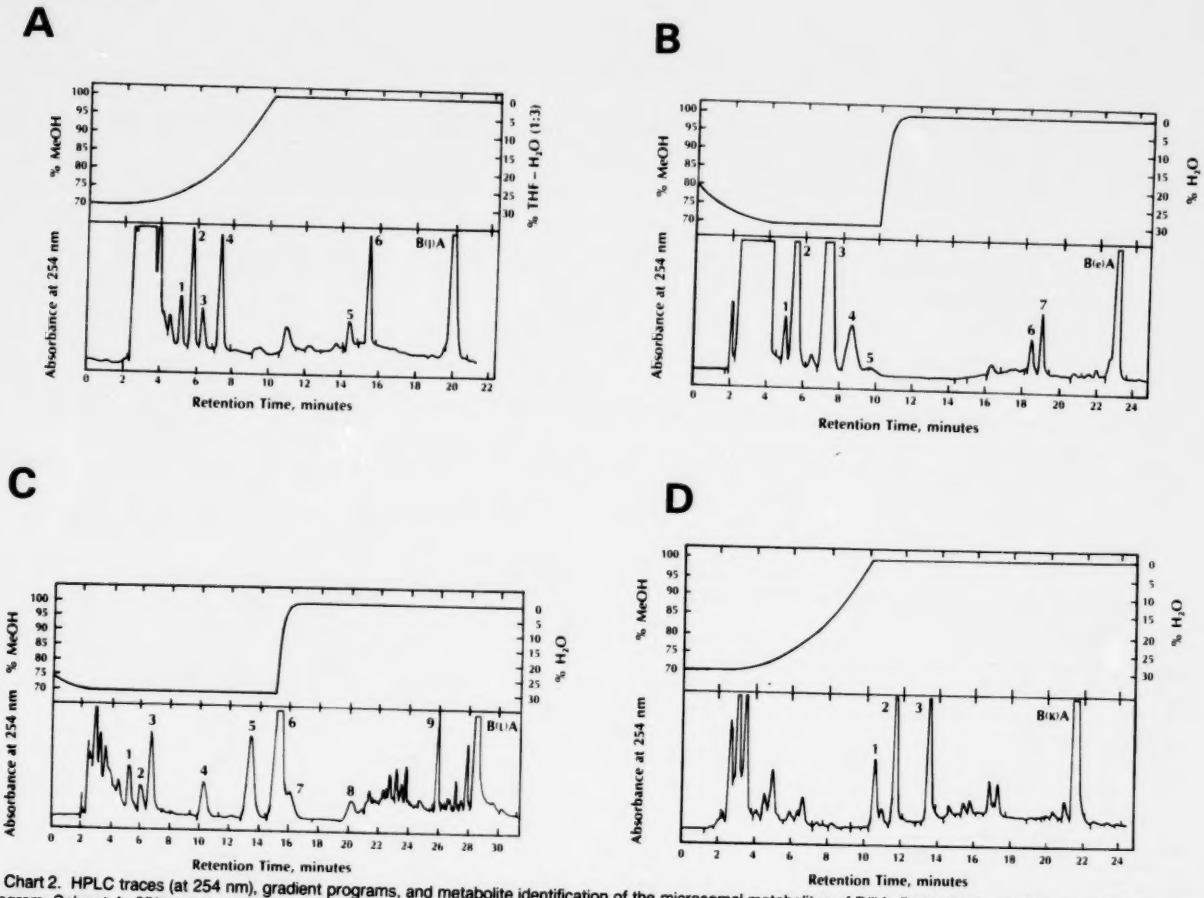



Chart 2. HPLC traces (at 254 nm), gradient programs, and metabolite identification of the microsomal metabolites of B(j)A, B(e)A, B(l)A, and B(k)A. *A*, B(j)A, gradient program: Solvent A, 25% tetrahydrofuran-water; Solvent B, methanol; flow rate, 2 ml/min; gradient segment 1: initial solvent, 70% Solvent B in A; final solvent, 100% Solvent B; gradient exponent, 3; gradient time, 10 min; gradient segment 2: 100% Solvent B, 12 min; Peak 1, B(j)A-11,12-dihydrodiol [B(j)$A_1$]; Peak 2, *trans*-B(j)A-1,2-dihydrodiol [B(j)$A_2$]. Synthetic *trans*-B(j)A-1,2-dihydrodiol chromatographs with Peak 2; Peak 4, B(j)A-9,10-dihydrodiol [B(j)$A_4$]; Peak 6, 10-hydroxy-B(j)A [B(j)$A_6$]. *B*, B(e)A; gradient program: Solvent A, water; Solvent B, methanol; flow rate, 2 ml/min; gradient segment 1: initial solvent, 80% Solvent B in A; final solvent, 70% Solvent B in A; gradient exponent, −5; gradient time, 10 min; gradient segment 2: initial solvent, 70% Solvent B in A; final solvent, 100% Solvent B; gradient exponent, −9; gradient time, 3 min; gradient segment 3: 100% Solvent B, 14 min; Peak 2, *trans*-B(e)A-5,6-dihydrodiol [B(e)$A_2$]. Synthetic *trans*-B(e)A-5,6-dihydrodiol cochromatographs with Peak 2; Peak 3, B(e)A-3,4-dihydrodiol [B(e)$A_3$]; Peak 4, B(e)A-1,2-dihydrodiol [B(e)$A_4$]. *C*, B(l)A; gradient program: Solvent A, water; Solvent B, methanol; flow rate, 2 ml/min; gradient segment 1: initial solvent, 75% Solvent B in A; final solvent, 70% Solvent B in A; gradient exponent, −5; gradient time, 5 min: gradient segment 2: 70% Solvent B in A, 10 min; gradient segment 3: initial solvent, 70% Solvent B in A; final solvent, 100% Solvent B; gradient exponent, −9; gradient time, 3 min; gradient segment 4: 100% Solvent B, 12 min. Peak 3, *anti-trans*-B(l)A-1,2-dihydrodiol [B(l)$A_3$]. Synthetic *anti-trans*-B(l)A-1,2-dihydrodiol cochromatographs with Peak 3; Peak 5, B(l)A-4,5-dihydrodiol [B(l)$A_5$]; Peak 6, B(l)A-7,8-dihydrodiol [B(l)$A_6$]. *D*, B(k)A; gradient program: Solvent A, water; Solvent B, methanol; flow rate, 2 ml/min; gradient segment 1: initial solvent, 70% Solvent B in A; final solvent, 100% Solvent B; gradient exponent, 3; gradient time, 10 min; gradient segment 2: 100% Solvent B, 12 min. Peak 2, B(k)A-4,5-dihydrodiol [B(k)$A_2$]; Peak 3, B(k)A-8,9-dihydrodiol [B(k)$A_3$].

been assigned structures and their relative yields are estimated to be B(j)$A_2 \gg$ B(j)$A_4$ > B(j)$A_6$ > B(j)$A_1$. The principal metabolite B(j)$A_2$ has been identified as *trans*-B(j)A-1,2-dihydrodiol. Its UV absorption spectrum resembles that of benz(*a*)anthracene (Chart 3). $^1$H NMR resonances ($H_6$, $H_7$) characteristic of aromatic bay-region protons are present, while absence of vinylic resonances rules out partial saturation of the terminal or angular benzo rings and absence of a cyclopenta-ring AX quartet precludes a K-region dihydrodiol formulation (Table 3). Absolute confirmation of this structure is provided by comparison with a synthetic standard. All spectral and chromatographic data from B(j)$A_2$ and synthetic *trans*-B(j)A-1,2-dihydrodiol were identical. Metabolite B(j)$A_1$ was assigned the K-region dihydrodiol structure [B(j)A-11,12-dihydrodiol] and is distinguished from B(j)$A_2$ by its UV absorption spectrum (Chart 3), which does not arise from the benz(*a*)anthracene chromophore. Proton resonances from solvent and HPLC column impurities, which appear as major signals because of the low concentration of B(j)$A_1$, obscure the region where AX quartet of the intact cyclopenta-ring should be observed. However, other features of the $^1$H NMR spectrum are consistent with a K-region dihydrodiol, *i.e.*, a low-field singlet and doublet ($H_6$, $H_7$), indicating an aromatic bay-region periphery, and absence of vinylic resonances precluding terminal or angular benzo-ring metabolism (Table 3). Characterization of B(j)$A_4$ as B(j)A-9,10-dihydrodiol rests partly upon the similarity of its UV absorption spectrum to that of anthracene (Chart 3), consistent with disruption of angular benzo-ring aromaticity. A crucial feature of the $^1$H NMR spectrum is the presence 2 vinyl resonances ($H_7$, $H_8$), since a possible 4,5-dihydrodiol would have only a single vinylic signal (Table 3). The marked downfield position of one of the vinyl signals ($H_7$) is consistent with location of a vinyl proton in the bay indentation, deshielded by the ring current of the

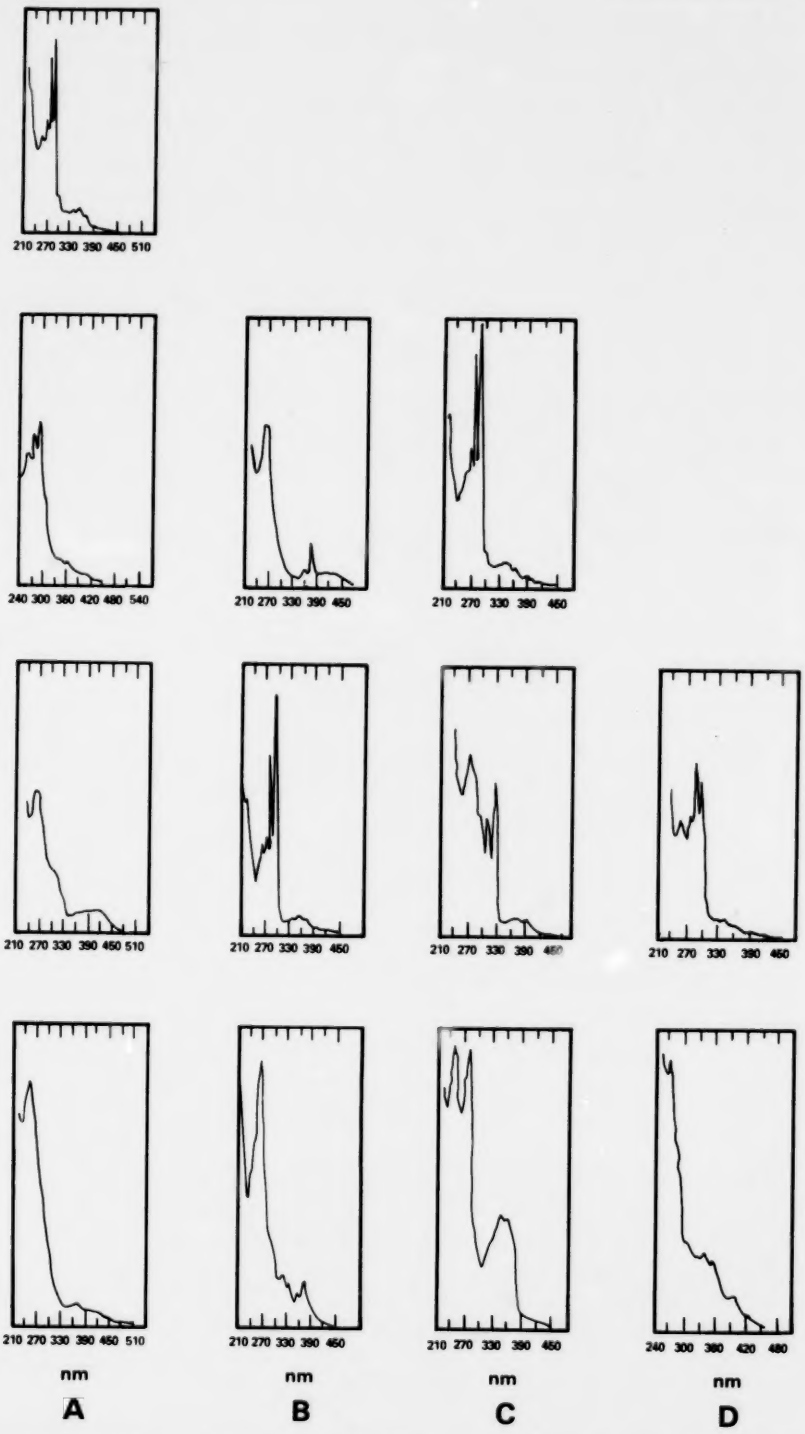



Chart 3. UV absorption spectra (methanol) of the major metabolites of cyclopenta-fused benz(*a*)-anthracenes. *A* (*top to bottom*), $B(j)A_2$, $B(j)A_1$, $B(j)A_6$, $B(j)A_4$; *B*, $B(e)A_4$, $B(e)A_2$, $B(e)A_3$; *C*, $B(l)A_3$, $B(l)A_5$, $B(l)A_6$; *D*, $B(k)A_2$, $B(k)A_3$. *Ordinate*, absorbance.

adjacent residual anthracene nucleus. $B(j)A_6$ was recognized as a phenol by its relative HPLC retention time, molelcular weight, elemental composition, and the long-wavelength band in the UV absorption spectrum indicating preservation of the B(j)A nucleus (Chart 3). Positional isomerism was established by analysis of the first-order couplings of the $^1H$ NMR spectrum (Table 3). Absence of a downfield aromatic AB or AX quartet rules out substitution of the terminal ring. The presence of the 2 highly deshielded bay-region signals is inconsistent with a $C_6$- or $C_7$-phenol, while substitution of the K-region would have resulted in

Table 3

*Cyclopenta-fused benz(a)anthracene metabolite proton shifts and coupling constants*

For data shown, $^1$H NMR is at 250 MHz, 25° (acetone-$d_6$).

| Metabolite | Proton, shift (ppm), (multiplicity[a]), coupling constants |
|---|---|
| B(j)$A_1$ | $H_3$, 7.95 (d), $J_{3,4}$ = 8.3 Hz; $H_5$, 8.37 (d), $J_{5,4}$ = 7.5 Hz; $H_6$, 9.20 (s); $H_7$, 8.96 (d), $J_{7,8}$ = 7.5 Hz; $H_{11}$, 5.48 (bs); $H_{12}$, 5.75 (bs); remaining aromatic H, 7.4–7.8 (m) |
| B(j)$A_2$ | $H_1$, 5.88 (bs); $H_2$, 5.56 (bs); $H_3$, 8.05 (d), $J_{3,4}$ = 8.3 Hz; $H_5$, 8.35 (d), $J_{5,4}$ = 8.3 Hz; $H_6$, 9.33 (s); $H_7$, 9.00 (d), $J_{7,8}$ = 7.3 Hz; remaining aromatic H, 7.5–8.0 (m) |
| B(j)$A_4$ | $H_1$, 7.73 (d), $J_{1,2}$ = 5 Hz; $H_3$, 8.10 (d), $J_{3,4}$ = 8.7 Hz; $H_5$, 8.35 (d), $J_{5,4}$ = 8.3 Hz; $H_6$, 8.96 (s); $H_7$, 7.49 (dd), $J_{7,8}$ = 11.3 Hz, $J_{7,9}$ = 2 Hz; $H_8$, 6.32 (dd), $J_{8,7} = J_{7,8}$ = 11.3 Hz, $J_{8,9}$ = 1.2 Hz; $H_9$, 4.55 (bd), $J_{9,10}$ = 11.3 Hz; $H_{10}$, 4.93 (bd), $J_{10,9} = J_{9,10}$ = 11.3 Hz; remaining aromatic H, 7.60–8.05 (m) |
| B(j)$A_6$ | $H_1$, 7.73 (d), $J_{1,2}$ = 5.3 Hz; $H_2$, 7.20 (d), $J_{2,1} = J_{1,2}$ = 5.3 Hz; $H_3$, 7.88 (d), $J_{3,4}$ = 7.0 Hz; $H_4$, 6.67 (dd), $J_{4,3} = J_{3,4}$ = 7.0 Hz, $J_{4,5}$ = 7.8 Hz; $H_5$, 8.14 (d), $J_{5,4} = J_{4,5}$ = 7.8 Hz; $H_6$, 9.40 (s); $H_7$, 4.47 (d), $J_{7,8}$ = 7.8 Hz; $H_8$, 7.53 (dd), $J_{7,8} = J_{8,7} = J_{8,9}$ = 7.8 Hz; $H_9$, ~7.1; $H_{11}$, 8.19 (d), $J_{11,12}$ = 8.8 Hz; $H_{12}$, 8.30 (d), $J_{12,11} = J_{11,12}$ = 8.8 Hz |
| B(e)$A_2$ | $H_1$, 8.54 (dd), $J_{1,2}$ = 6.2 Hz, $J_{1,3}$ = 4.2 Hz; $H_4$, 8.28 (dd), $J_{4,3}$ = 6.3 Hz, $J_{4,2}$ = 5.2 Hz; $H_5$, 5.88 (bs); $H_6$, 5.47 (bs); $H_7$, 7.65 (s); $H_8$, 7.97 (dd), $J_{8,9}$ = 7.3 Hz, $J_{8,10}$ = 2.1 Hz; $H_{11}$, 8.86 (dd), $J_{11,10}$ = 7.3 Hz, $J_{11,9}$ = 2.3 Hz; remaining aromatic H, 7.58–7.70 (m) |
| B(e)$A_3$ | $H_1$, 5.15 (d), $J_{1,2}$ = 9.4 Hz; $H_2$, 4.54 (bd), $J_{2,1} = J_{1,2}$ = 9.4 Hz; $H_3$, 6.07 (dd), $J_{3,4}$ = 10.4 Hz, $J_{3,2}$ = 2.5 Hz; $H_4$, 6.97 (dd), $J_{4,3} = J_{3,4}$ = 10.4 Hz, $J_{4,2}$ = 2.0 Hz; $H_5$, 7.82 (d), $J_{5,6}$ = 5.3 Hz; $H_6$, 7.13 (d), $J_{6,5} = J_{5,6}$ = 5.3 Hz; $H_7$, 8.06 (s); $H_8$, 8.08 (d); $H_{11}$, 8.72 (d), $J_{11,10}$ = 7.3 Hz; $H_{12}$, 8.28 (s); $H_8$, $H_9$ (two dd), 7.70 and 7.12, $J_{8,9} = J_{9,10} = J_{10,11}$ = 7.3 Hz |
| B(e)$A_4$ | $H_1$, 7.21 (d), $J_{1,2}$ = 9.4 Hz; $H_2$, 6.33 (d), $J_{1,2} = J_{2,1}$ = 9.4 Hz; $H_3$, 4.57 (bd), $J_{3,4}$ = 10.4 Hz; $H_4$, 4.98 (d), $J_{4,3} = J_{3,4}$ = 10.4 Hz; $H_5$, 7.68 (d), $J_{5,6}$ = 5.2 Hz; $H_6$, 7.17 (d), $J_{6,5} = J_{5,6}$ = 5.2 Hz; $H_7$, 8.22 (s); $H_8$, 8.20 (d); $H_{11}$, 8.36 (d), $J_{11,10}$ = 8.3 Hz; $H_{12}$, 8.88 (s); $H_8$, $H_9$, 7.46–7.67 (m) |
| B(l)$A_3$ | $H_1$, 6.12 (bs); $H_2$, 5.56 (bs); $H_6$, 8.47 (s); $H_{12}$, 9.40 (d), $J_{12,11}$ = 7.5 Hz; remaining aromatic H, 7.60–8.07 (m) |
| B(l)$A_5$ | $H_1$, 8.21 (d), $J_{1,2}$ = 5.3 Hz; $H_2$, 6.86 (d), $J_{2,1} = J_{1,2}$ = 5.3 Hz; $H_3$, 6.71 (d), $J_{3,4}$ = 3.0 Hz; $H_4$, 4.96 (dd), $J_{4,5}$ = 8.4 Hz; $H_5$, 5.15 (d), $J_{5,4} = J_{4,5}$ = 8.4 Hz; $H_6$, 7.90 (s); $H_{12}$, 9.03 (d), $J_{12,11}$ = 7.8 Hz; remaining aromatic H, 7.57–8.04 (m) |
| B(l)$A_6$ | $H_1$, 7.77 (d), $J_{1,2}$ = 4.8 Hz; $H_2$, 7.43 (d), $J_{2,1} = J_{1,2}$ = 4.8 Hz; $H_6$, 8.42 (s); $H_7$ and $H_8$, 4.91, 4.89, AB quartet, $J_{AB}$ = 9.2 Hz; remaining aromatic H, 7.66–8.12 (m) |
| B(k)$A_2$ | $H_1$, 8.69 (d), $J_{1,2}$ = 7.5 Hz; $H_4$, 5.36 (bs); $H_5$, 5.90 (bs); $H_6$, 7.80 (s); $H_7$, 8.53 (s); $H_{12}$, 9.30 (s); remaining aromatic H, 7.50–7.70 (m) |
| B(k)$A_3$ | $H_1$, 8.54 (d); $H_4$, 7.20 (d), $J_{4,5}$ = 5.0 Hz; $H_5$, 7.29 (d), $J_{5,4} = J_{4,5}$ = 5.0 Hz; $H_6$, 8.17 (s), $H_7$, 8.30 (s); $H_8$, 4.91 (d), $J_{8,9}$ = 10.0 Hz, $H_9$, 4.55 (bd), $J_{9,8} = J_{8,9}$ = 10.0 Hz; $H_{10}$, 6.77 (dd), $J_{10,11}$ = 10.0 Hz, $J_{10,9}$ = 2.5 Hz; $H_{11}$, 6.17 (dd), $J_{11,10} = J_{10,11}$ = 10.0 Hz, $J_{11,9}$ = 2.5 Hz; $H_{12}$, 8.53 (s); remaining aromatic H, 7.68–7.80 (m) |

[a] Multiplets are designated as follows: *s*, singlet; *bs*, broadened singlet; *d*, doublet; *bd*, broadened doublet; *dd*, doublet of doublets; *m*, complex multiplet.

the presence of a second aromatic singlet. Substitution at $C_8$ or $C_9$ would result in an upfield shift of 2 adjacent aromatic protons by the "ortho effect" (31). Integration of the aromatic resonances shows that only one aromatic proton can be shifted upfield ($H_9$, obscured by the impurity at 7 to 7.2 ppm); hence, substitution at $C_{10}$ [10-hydroxy-B(j)A] is compatible with the $^1$H NMR spectrum.

**B(e)A.** Metabolites B(e)$A_2$, B(e)$A_3$, and B(e)$A_4$ have been assigned structures (Chart 2), with relative abundances estimated to be B(e)$A_2$ ≈ B(e)$A_3$ > B(e)$A_4$. B(e)$A_2$ was identified as *trans*-B(e)A-5,6-dihydrodiol by its benz(*a*)anthracene-like UV absorption spectrum (Chart 3) and by the absence of both vinylic resonances and a cyclopenta-ring AX quartet in the $^1$H NMR (Table 3). Absolute confirmation of the structure is provided by comparison with a synthetic standard. All spectral and chromatographic data from B(e)$A_2$ and synthetic *trans*-B(e)A-5,6-dihydrodiol were identical. B(e)$A_3$ and B(e)$A_4$ were identified as terminal ring dihydrodiols by the similarity of their UV absorption spectra to that of acephenanthrylene (Chart 3) (11, 26, 27), and the presence of aromatic bay-region signals in the $^1$H NMR (Table 3). The *meso* bay-region proton resonance ($H_{12}$) of the isomer with the dihydrodiol distal to the bay region is expected to be shifted upfield relative to the resonance of the bay-region proton ($H_{11}$) on the angular ring (13). On this basis, B(e)$A_3$ is B(e)A-3,4-dihydrodiol, and B(e)$A_4$ is B(e)A-1,2-dihydrodiol.

**B(l)A.** Metabolites B(l)$A_3$, B(l)$A_5$, and B(l)$A_6$ have been identified (Chart 2), and their relative amounts have been estimated to be B(l)$A_6$ > B(l)$A_3$ ≫ B(l)$A_5$. B(l)$A_3$ was identified as *anti-trans*-B(l)A-1,2-dihydrodiol from its benz(*a*)anthracene-like UV absorption spectrum (Chart 3) and by the absence both of vinylic proton resonances and of a cyclopenta-ring AX quartet in the $^1$H NMR (Table 3). Severe steric crowding within the gulf region requires

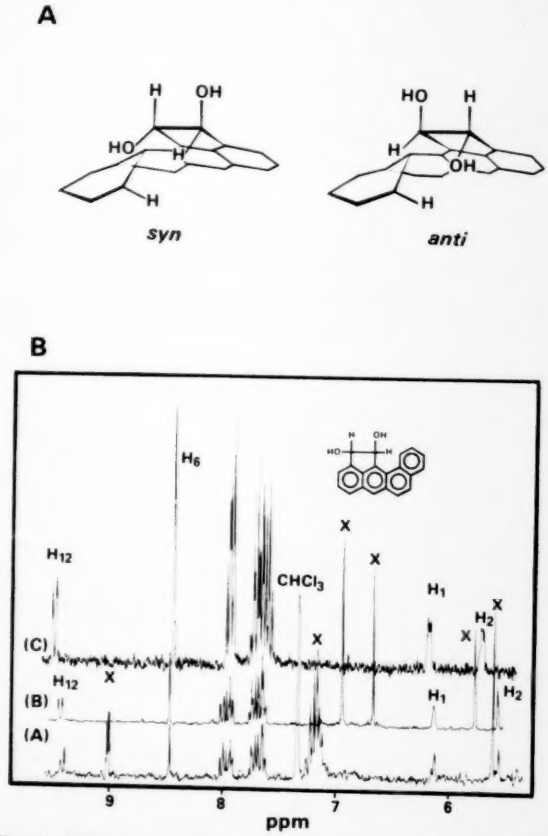



Chart 4. Configuration of the 2 possible *trans*-1,2-dihydrodiols of B(l)A and $^1$H NMR of metabolite B(l)$A_3$ and synthetic *trans*-B(l)A-1,2-dihydrodiols. *A*, absolute configuration of 2 isomers B(l)$A_{3a}$ (*syn*) and B(l)$A_{3b}$ (*anti*). *B*, $^1$H NMR (250 MHz, acetone-$d_6$, 29°) of: Scan A, metabolite B(l)$A_3$; Scan B, synthetic *anti-trans*-B(l)A-1,2-dihydrodiol [B(l)$A_{3b}$]; Scan C, synthetic *syn-trans* B(l)A-1,2-dihydrodiol [B(l)$A_{3a}$]. ×, resonances arising from impurities. Note the correspondence of the chemical shifts of $H_{12}$, $H_1$, and $H_2$ between metabolite B(l)$A_3$ and reference standard B(l)$A_{3b}$ and the patterns of the multiplets from 7.8 to 8.0 ppm.

B(l)$A_3$ to adopt a nonplanar configuration. As a result, isomeric (*syn*- and *anti*-) *trans*-dihydrodiols B(l)$A_{3a}$ and B(l)$A_{3b}$ are possible (Chart 4*A*). The 2 isomeric dihydrodiols have been synthesized, resolved by HPLC, and characterized. Structural assignments are based on the reasonable assumptions that *m*-chloroperoxybenzoic acid oxidation of B(l)A yields an epoxide with the oxirane ring oriented *anti*- to the gulf region and that subsequent hydration by potassium hydroxide involves $S_N2$ attack of hydroxide ion at $C_1$ to yield the *syn* dihydrodiol. The striking difference in retention times of B(l)$A_{3a}$ and B(l)$A_{3b}$ may be explained by an expected decrease in polarity when the $C_1$ hydroxy group is located within the gulf. The HPLC retention times are therefore in accord with the assignment of the *syn* configuration to product B(l)$A_{3a}$ obtained by $S_N2$ hydration of the putative *anti*-epoxide. Surprisingly, action of epoxide hydrolase on the 1,2-oxide quantitatively yielded dihydrodiol B(l)$A_{3b}$, indicating enzymic hydration occurs at the less reactive, but also less hindered $C_2$ position (data not shown). The isolated metabolite corresponds by HPLC retention time (see "Materials and Methods") and $^1$H NMR (Chart 4*B*, Scan *A*) to the *anti-trans*-isomer B(e)$A_{3b}$ (Scan *B*) rather than the *syn-trans* isomer B(l)$A_{3a}$ (Scan *C*).

The UV absorption spectrum of B(l)$A_5$ suggests that the terminal benzo-ring has been partially saturated, leaving a phenanthrene-like aromatic nucleus (Chart 3). The most likely formulation for a terminal ring dihydrodiol is B(l)A-4,5-dihydrodiol, and this structure is confirmed by the $^1$H NMR (Table 3). The critical feature is the presence of only a single vinyl resonance split by allylic coupling to a carbinyl proton.

Metabolite B(l)$A_6$ is the K-region dihydrodiol, B(l)A-7,8-dihydrodiol. The UV absorption spectrum indicates a chromophore different from benz(*a*)anthracene (Chart 3) and the $^1$H NMR, while having no vinylic signals, exhibits the AX quartet of the unsaturated cyclopenta ring (Table 3). The carbinyl protons appear as a true AB quartet, typical of the similar magnetic environments of K-region positions (19).

**B(k)A.** Two metabolites, B(k)$A_2$ and B(k)$A_3$, were collected in sufficient quantity for characterization (Chart 2). B(k)$A_2$ was the predominant metabolite. The UV absorption spectrum of B(k)$A_2$, while differing somewhat from that of benz(*a*)anthracene, was identical to that of 4,5-dihydro-B(k)A (Chart 3); hence, B(k)$A_2$ is the cyclopenta-ring dihydrodiol, B(k)A-4,5-dihydrodiol. The $^1$H NMR substantiates the assignment, since vinyl resonances and a cyclopenta-ring AX quartet are absent, and 2 carbinyl protons account for the only nonaromatic signals in the spectrum (Table 3). The UV absorption spectrum of B(k)$A_3$ (Chart 3) suggests partial saturation of the terminal benzo ring. The structure B(k)A-8,9-dihydrodiol is assigned on the basis of the upfield chemical shift of the *meso* bay-region proton with respect to $H_1$ by the same rationale (13) applied to the structural assignments of metabolites B(e)$A_3$ and B(e)$A_4$ (Table 3).

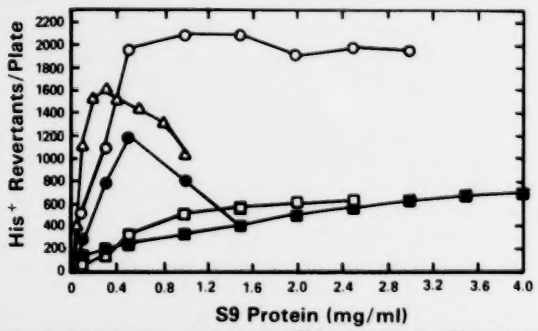



Chart 5. Relationship between Aroclor 1254-induced rat liver S9 concentration and mutagenic activity of B(j)A, B(e)A, B(l)A, and B(k)A in strain TA98 of *S. typhimurium*. The amount of substrate was 30 μg/plate. The background mutation frequencies were 36 histidine revertants/plate (average). Data represent the mean of triplicate determinations. ○, B(e)A; ●, B(l)A; △, B(j)A; □, B(k)A; ■, B(a)P.

**Mutagenic Activity of Cyclopenta-fused PAH in *S. typhimurium*.** Previous studies with cyclopenta(*cd*)pyrene and B(j)A had shown a sharp dependence of mutagenic response on S9 concentration in bacterial (15, 38) assays. Similar studies were performed with all 4 cyclopenta-fused benz(*a*)anthracene isomers and B(a)P in TA98 (Chart 5). The mutagenic activity of 2 of the 4 isomers, B(l)A and B(j)A, exhibited a strong dependence on S9 protein concentration, with optimal activity observed at 0.5- and 0.3-mg/ml S9 protein, respectively. Higher concentrations of S9 resulted in markedly diminished activity. The mutagenic activity of B(e)A exhibited a plateau at 0.5-mg/ml S9 protein and retained that level up to 3.0 mg/ml. Higher concentrations of S9 protein were required for B(k)A and B(a)P activity to reach a plateau; the range was 1.0 to 1.5 mg/ml for B(k)A and 3.0 to 3.5 mg/ml for B(a)P. Comparison of the 5 PAH at their optimum S9 concentrations indicated that B(e)A > B(j)A > B(l)A ≫ B(k)A,B(a)P (Chart 5) based on the maximum number of revertants per plate. The sensitivity of the 4 cyclopenta-fused PAH to *S. typhimurium* strain differences was also examined. All were active in strains TA1538, TA98, and TA100 and inactive in TA1535 and TA1537, except for B(k)A and B(e)A which were active in TA1537 (data not shown).

**Mutagenic Activity of Cyclopenta-fused PAH in V79 Cells.** Induced gene mutation in V79 cells was measured at the hypoxanthine-guanine phosphoribosyltransferase locus (6TG-resistance) using an Aroclor 1254-induced liver S9 from male Charles River rats as the metabolic activation system. The dependence of mutagenic activity on S9 protein concentration (0 to 3 mg/ml) was examined at PAH concentrations of 1, 5, 10 μg/ml (Charts 6 and 7). B(e)A, B(l)A, B(j)A, B(k)A, and B(a)P were mutagenic at 5 and 10 μg/ml but had marginal or no activity at 1 μg/ml even at S9 protein concentrations as high as 3.0 mg/ml. Mutagenic activity appeared to plateau at S9 protein concentrations of 0.5 mg/ml or less for B(l)A, B(e)A, B(j)A, and B(a)P. With a 0.5-mg/ml protein concentration, the 5 PAH were examined at a concentration range of 0 to 20 μg/ml (Chart 8). At 20 μg/ml, B(l)A was the most active, and the remaining PAH were equivalent in activity to B(a)P.

Irradiated SHE cells were examined as an alternate metabolic activation system for V79 cells. Preliminary SHE cell density experiments suggested $2.0 \times 10^6$ SHE cells to be optimum for B(a)P activity. B(j)A, B(e)A, and B(k)A did not induce gene mutation in V79 cells when cocultivated with SHE cells in the concentration range 0 to 2.0 μg/ml, where significant cytotoxicity was evident at the highest concentrations. Experiments performed at lower SHE cell densities ($0.25 \times 10^6$, $0.5 \times 10^6$, or $1.0 \times 10^6$) also indicated no genetic activity (data not shown). However, B(l)A was quite active, producing 635 6TG-resistant mutants/$10^6$ cells, while B(a)P, under similar conditions and at equimolar concentrations, produced 1178 6TG-resistant mutants/$10^6$ survivors (Chart 9).

**Mutagenic Activity of B(l)A-1,2-oxide.** The mutagenic activity of B(l)A-1,2-oxide was compared to *anti*-B(a)P-7,8-dihydrodiol-9,10-oxide and *syn*-B(a)P-7,8-dihydrodiol-9,10-oxide in both *S. typhimurium* and V79 cells without exogenous metabolic acti-

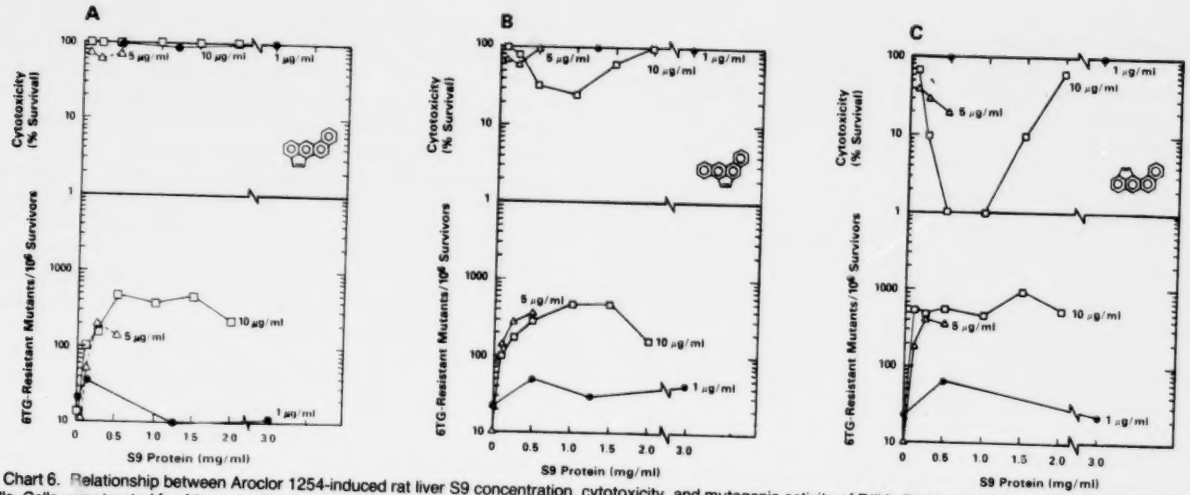



Chart 6. Relationship between Aroclor 1254-induced rat liver S9 concentration, cytotoxicity, and mutagenic activity of B(j)A, B(e)A, and B(l)A in Chinese hamster V79 cells. Cells were treated for 4 hr and subsequently scored for cytotoxicity and 6TG-resistance according to "Materials and Methods." Data represent the mean of triplicate determinations. *A*, B(j)A; *B*, B(e)A; *C*, B(l)A.

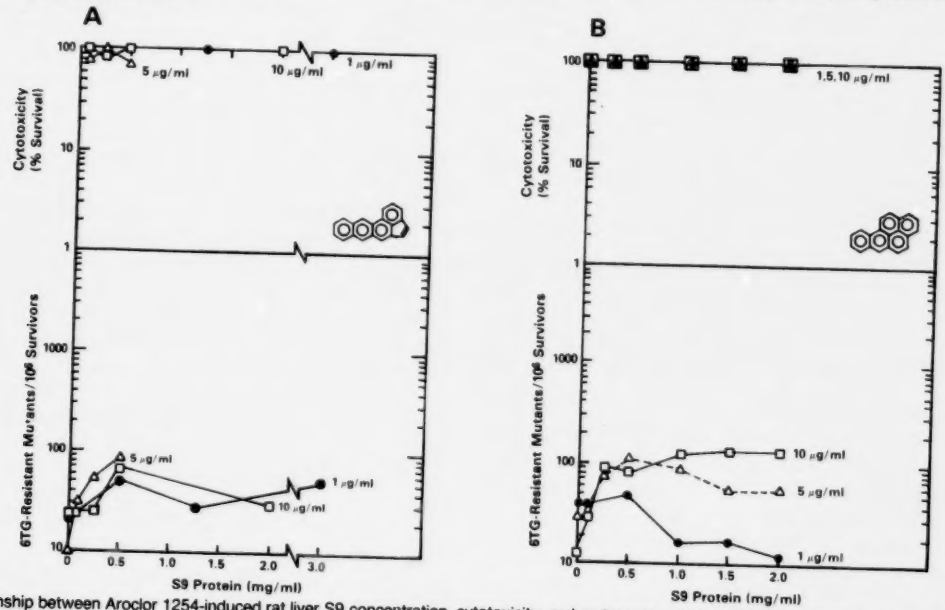



Chart 7. Relationship between Aroclor 1254-induced rat liver S9 concentration, cytotoxicity, and mutagenic activity of B(k)A and B(a)P in Chinese hamster V79 cells. Cells were treated for 4 hr and subsequently scored for cytotoxicity and 6TG-resistance according to "Materials and Methods." *Data points*, mean of triplicate determinations. *A*, B(k)A; *B*, B(a)P.

vation. All 3 epoxides were active as bacterial mutagens; *syn*-B(a)P-7,8-dihydrodiol-9,10-oxide produced more histidine revertants than the *anti* isomer and slightly less than B(l)A-1,2-oxide (Chart 10). However, on a molar basis, 11-fold more B(l)A-1,2-oxide was required than *syn*-B(a)P-7,8-dihydrodiol-9,10-epoxide to yield an equivalent number of revertants.

B(l)A-1,2-oxide was also active as a gene mutagen in V79 cells producing a concentration-related response of 320 6TG-resistant mutants/$10^6$ survivors at 1.0 μg/ml (Chart 11). Both diol-epoxides of B(a)P were active, with the *anti*-isomer more than 2-fold as potent as the *syn*.

## DISCUSSION

Large values of $\Delta E_{deloc}/\beta$ (>0.79) for benzylic carbonium ions derived from ring-opened epoxides appear to be associated with biological activity (12, 24). Consequently, the large delocalization energies of carbonium ions from the cyclopenta-ring epoxides of B(j)A, B(e)A, and B(l)A suggest that if epoxidation of the cyclopenta-ring is an important metabolic pathway, these compounds should be active. On the other hand, application of the same criterion to B(k)A indicates that the 5-membered ring may not be a potential site for activation. Elucidation of the metabolite profiles of the 4 isomers demonstrates that epoxidation of the cyclopenta-fused ring is indeed a major pathway. The mutagenicity assays verify the prediction of biological activity for B(j)A, B(e)A, and B(l)A and support the importance of large $\Delta E_{deloc}/\beta$.

Additional potentially active metabolites of B(j)A and B(l)A have been identified and may also contribute to total mutagenic response. B(j)A-9,10-dihydrodiol is capable of further metabolic

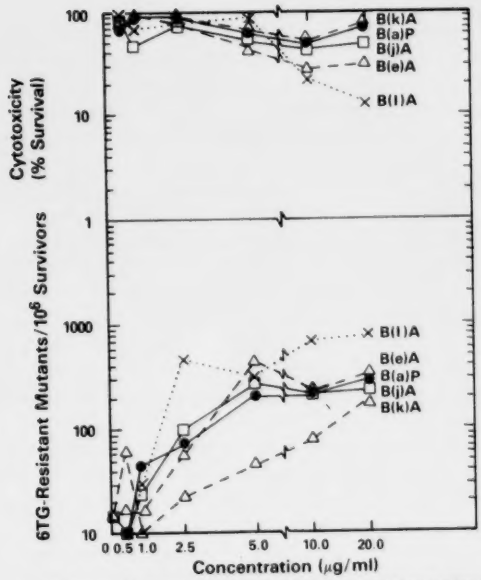



Chart 8. Induction of 6TG resistance and cytotoxicity in V79 cells after treatment with B(j)A, B(e)A, B(l)A, B(k)A, and B(a)P. V79 cells were treated with PAH and an Aroclor 1254-induced rat liver S9 (0.5 mg/ml) for 4 hr. Data represent the average of duplicate determinations.

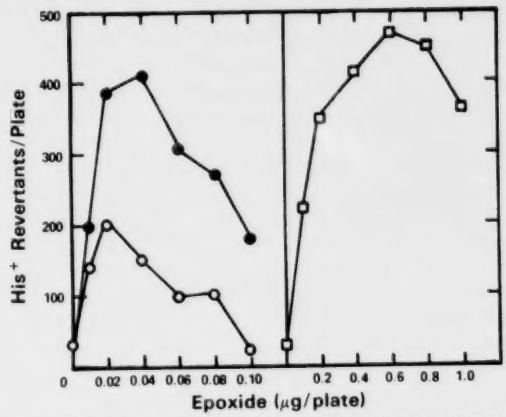



Chart 10. Mutagenicity of B(l)A-1,2-oxide, *syn*-B(a)P-7,8-diol-9,10-epoxide, and *anti*-B(a)P-7,8-diol-9,10-epoxide in strain TA98 of *S. typhimurium*. The epoxides were added in 12.5 μl of anhydrous dimethyl sulfoxide to 0.5 ml of PBS, pH 7.0, which contained $2 \times 10^8$ bacteria. After 5 min at 37°, 2 ml of the molten top agar were added to the incubation mixture, and the solution was poured into Petri dishes as described in "Materials and Methods." Each value represents the average of duplicate determinations. ●, *syn*-B(a)P-7,8-diol-9,10-epoxide; ○, *anti*-B(a)P-7,8-diol-9,10-epoxide; □, B(l)A-1,2-oxide.

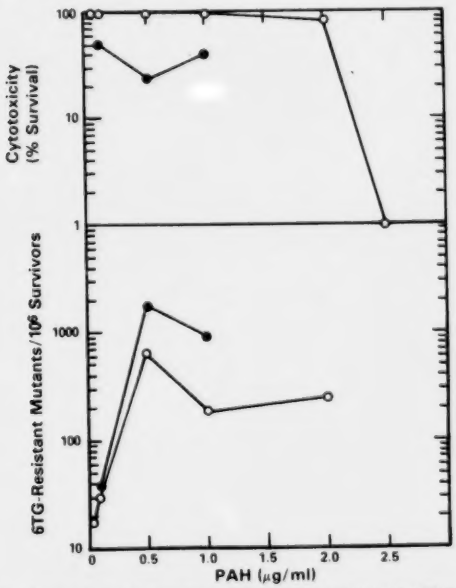



Chart 9. Mutagenic and cytotoxic activity of B(l)A and B(a)P in V79 cells cocultivated with irradiated SHE cells. V79 cells ($3 \times 10^5$/dish) seeded on irradiated SHE cells ($2 \times 10^6$/dish) were incubated with B(l)A or B(a)P for 48 hr. V79 cells were then separated from the SHE cells, and the induction of 6TG resistance and cytotoxicity was determined. Each *point* represents the mean of 4 replicate experiments. ○, B(l)A; ●, B(a)P.

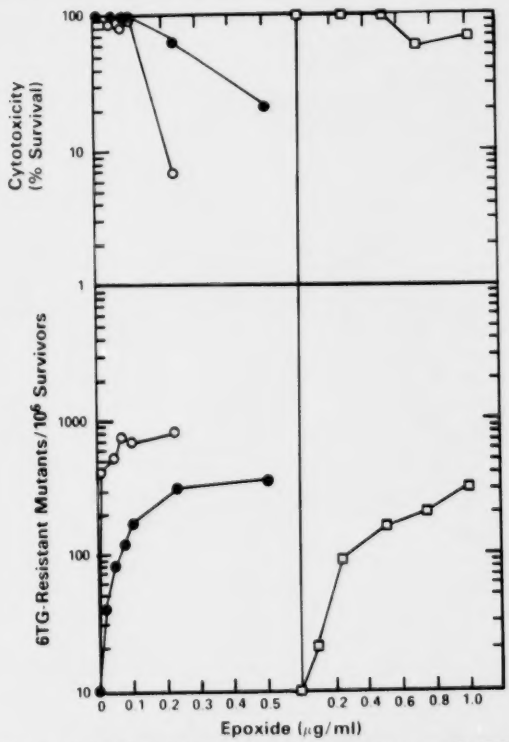



Chart 11. Mutagenicity and cytotoxicity of B(l)A-1,2-oxide, *syn*-B(a)P-7,8-diol-9,10-epoxide, and *anti*-B(a)P-7,8-diol-9,10-epoxide in Chinese hamster V79 cells. Epoxides dissolved in anhydrous dimethyl sulfoxide were added to V79 cells which were seeded at a density of $1 \times 10^6$ cells/dish, 18 hr previously. Exposure time was 4 hr at 37°. The V79 cells were subsequently scored for cytotoxicity and induction of 6TG-resistant mutants. Each value represents the average of duplicate determinations. ●, *syn*-B(a)P-7,8-dihydrodiol-9,10-epoxide; ○, *anti*-B(a)P-7,8-dihydrodiol-9,10-epoxide; □, B(l)A-1,2-oxide.

transformation to a bay-region diol-epoxide, while the isolation of K-region dihydrodiols of B(j)A and B(l)A imply the presence of the corresponding potentially active K-region oxides. However, the K-region oxide of B(j)A is a minor metabolite and, for reasons discussed below, B(j)A-9,10-dihydrodiol does not appear to be an important promutagen. The K-region dihydrodiol is the major B(l)A metabolite isolated, but the high level of direct-acting mutagenicity of B(l)A-1,2-oxide, which is also a major metabolite, is consistent with a role for the 1,2-oxide in the activity of B(l)A.

In *S. typhimurium*, the peak mutagenic activity of B(j)A, B(e)A,

and B(l)A occurred at considerably lower S9 protein concentrations (0.3 to 0.5 mg/ml) than B(k)A and B(a)P (1.0 to 3.5 mg/ml), suggesting activation via different pathways for the 2 groups. A low optimum S9 protein concentration has been reported for cyclopenta(*cd*)pyrene (15) and interpreted as a one-step cyclopenta-ring epoxide activation pathway in contrast to the multistep diol-epoxide pathway of B(a)P. The broad curve of the dependence of bacterial mutagenicity on S9 protein concentration for B(a)P is intrepreted as the result of a combination of activation and deactivation curves resulting from the formation of the 2 major contributors to the genetic activity; B(a)P-4,5-oxide and B(a)P-diol epoxides. The similarity between S9 dependence curves of cyclopenta(*cd*)pyrene, B(j)A, B(e)A, and B(l)A strongly support involvement of one-step epoxidation of the cyclopenta-ring as demonstrated in the case of cyclopenta(*cd*)pyrene. The direct-acting mutagenicity of *anti*-B(l)A-1,2-oxide, which accounts for up to 40% of the response of B(l)A in *S. typhimurium* and V79 cells, provides additional support for this contention.

A possible explanation for the contrasting S9 dependence observed for B(k)A is that B(k)A-8,9-dihydrodiol may contribute to activity via further metabolism to a mutagenic B(k)A-8,9-dihydrodiol-10,11-oxide, in a manner analogous to benz(*a*)anthracene (43, 45). However, confirmation of this route awaits the identification of tetrol and triol products of secondary metabolism of the B(k)A-8,10-dihydrodiol. Also, additional as yet unidentified primary metabolites present in the incubation might be involved in mutagenic activity. Nevertheless, multiple pathways appear to be involved in the activity of B(k)A whose cyclopenta-ring oxide does not open to as highly stabilized a carbonium ion as B(a)P-7,8-dihydrodiol-9,10-epoxide or the cyclopenta-epoxides of the benzaceanthrylene isomers.

Mutagenesis studies with B(j)A, B(e)A, B(l)A, and B(k)A in V79 cells do not reveal the same sensitivity to S9 protein concentration observed in the mutation response curves of *S. typhimurium*. In V79 cells, B(l)A proved to be the most active of the 4 isomers, comparable in mutagenicity to B(a)P.

Lack of mutagenicity of B(j)A, B(e)A, and B(k)A in SHE cell-V79 cell-mediated studies is of interest because most PAH are generally activated more efficiently by SHE cells than by rat liver S9 (28). Preliminary comparative studies on the quantitative metabolism of B(j)A by SHE cells and Aroclor 1254-induced rat liver S9 indicate that SHE cells produce twice as much B(j)A-9,10-dihydrodiol as B(j)A-1,2-dihydrodiol, while rat liver rapidly generates predominantly B(j)A-1,2-dihydrodiol (33). These results suggest that SHE cells can display regioselectivity for metabolism at sites other than cyclopenta-rings and that differences in activity obtained with different activating systems may, therefore, be partly ascribed to changes in regioselectivity of mixed-function oxidases.

This report suggests that B(l)A will serve as a valuable model for investigation of the balance between electronic and stereochemical factors in biological activity. Evidence clearly points to B(l)A-1,2-oxide as a major ultimate mutagen in both bacterial and V79 cell systems. The influence of both steric and electronic factors can be invoked to explain some of the behavior observed in this study. In the mammalian assays, B(l)A proved to be the most active cyclopenta-PAH despite the fact that the oxirane-derived carbonium ion has a smaller $\Delta E_{deloc}/\beta$ than either B(j)A or B(e)A. The severe steric crowding within the 4-sided gulf, which forces the molecule to adopt a nonplanar configuration, probably plays a role in enhancing activity. Similar steric interactions appear to be associated with enhanced activity in benzo(*c*)phenanthrene (23, 30) and bay-region PAH in which methyl substituents protrude into bay areas (21, 40).

Cyclopenta(*cd*)pyrene, like B(l)A, contains a fused cyclopenta-ring, and has been reported to be a strong bacterial mutagen in *S. typhimurium* (10, 44) but less active than B(a)P as a gene mutagen in V79 cells (35) and less active than B(a)P in mammalian cell morphological transformation (16). In contrast, B(j)A, B(e)A, and B(l)A are more active as gene mutagens than B(a)P in *S. typhimurium* and are as active or more active than B(a)P in V79 cells. We have recently reported that B(l)A is approximately 4-fold more active than B(a)P, and B(e)A as active as B(a)P as a mouse skin tumor initiator in SENCAR mice (32). These results are in sharp contrast to the mouse skin tumor-initiating activity of cyclopenta(*cd*)pyrene which is considerably less than B(a)P (6, 35, 44).

Benz(*a*)anthracene is reported to be weakly active as a gene mutagen in both the SHE cell-V79 cell and rat liver S9-V79 cell mutagenesis systems (25, 42) and weakly active as a mouse skin tumor initiator (42). Fusion of a cyclopenta-ring on benz(*a*)anthracene to give B(j)A, B(e)A, and B(l)A has produced dramatic increases in the ability of this angular PAH to induce gene mutation in bacteria and mammalian cells and in 2 examples [B(e)A and B(l)A] to initiate papilloma formation in mouse skin.

## ACKNOWLEDGMENTS

The authors thank Joye Denning and Hinda Bergman for their skillful preparation of this manuscript, and Dr. Eugene Elmore and Dr. George Hatch for the Syrian hamster embryo cells. We also thank the National Cancer Institute for their generous gift of the B(a)P standards.

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